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Localization of Alternatively Spliced NMDAR1 Glutamate Receptor Isoforms in Rat Striatal Neurons

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ABSTRACT

Alternative splicing of the mRNA encoding the *N*-methyl-D-aspartate (NMDA) receptor subunit NR1 changes the structural, physiologic, and pharmacologic properties of the resultant NMDA receptors. We used dual label immunocytochemistry and confocal microscopy to localize the four alternatively spliced segments of the NR1 subunit (N1, C1, C2, and C2′) in rat striatal neurons. Striatofugal projection neurons and four populations of interneurons were studied. Projection neurons, which were identified by immunolabeling for calbindin and by retrograde tracing from the globus pallidus and the substantia nigra, were the only striatal neurons containing C1 segment immunoreactivity. Projection neurons were also C2 segment immunopositive, as were all other neuronal populations studied. Projection neurons were C2′ segment immunonegative. In contrast, each of the interneuron types were labeled by the antibody to the C2′ segment: nitric oxide synthase interneurons were labeled intensely, calretinin and parvalbumin neurons were labeled moderately strongly, and cholinergic neurons were also labeled but less strongly than the other types of interneurons. Parvalbumin interneurons showed distinct N1 segment immunolabeling, which was not found in other types of striatal neurons. Our results suggest that all striatal neurons studied synthesize NR1 subunit proteins, but the isoforms of the protein present in projection neurons and the several types of interneurons are distinct. This differential expression of NR1 isoforms may affect both neuronal function and selective vulnerability of neurons to injury. J. Comp. Neurol. 415:204–217, 1999.

Indexing terms: glutamate receptors; projection neurons; interneurons; striatum; cholinergic; calbindin

The striatum is the primary target of afferent input to the basal ganglia (Albin et al., 1989; Graybiel, 1990; Parent and Hazrati, 1995). Medium-sized spiny neurons, which project to the pallidum, to the entopeduncular nucleus, and to the substantia nigra, constitute about 90% of the striatal neurons (Gerfen, 1992). In addition, there are several types of striatal interneurons present, with distinct morphologic and physiologic properties. Four of the populations of interneurons can be identified histochemically by the presence of (1) choline acetyltransferase (ChAT), (2) parvalbumin, (3) calretinin, and (4) neuronal nitric oxide synthase (nNOS; for review, see Kawaguchi et al., 1995). Projection neurons and, to a lesser extent, interneurons receive dense glutamatergic afferents from the neocortex (Divac et al., 1977; Kim et al., 1977; McGeer et al., 1977), the thalamus (Dubé et al., 1988), and the subthalamic nucleus (Kita and Kitai, 1987). Their excitatory input activates ionotropic *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate, and kainate receptors and metabotropic glutamate

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receptors (Tallaksen-Greene and Albin, 1994; Bernard et al., 1997; Götz et al., 1997; Bernard and Bolam, 1998; Testa et al., 1998).

NMDA receptor subunit messenger RNAs and proteins are abundant in the striatum (Petralia et al., 1994; Standaert et al., 1994; Kosinski et al., 1998), where the receptors serve a variety of functions. Striatal NMDA receptors are involved in the regulation of γ-aminobutyric acid (GABA), neuropeptide, and acetylcholine release (Damsma et al., 1991; Ruzicka and Jhamandas, 1991; Young and Bradford, 1991; Bustos et al., 1992; Galli et al., 1992; Somers and Beckstead, 1992; Morari et al., 1993; Young and Bradford, 1993), and their activation stimulates the expression of the immediate-early genes c-fos and c-jun (Xia et al., 1996; Berretta et al., 1997). NMDA activation causes striatal neurons to dephosphorylate and thereby to inactivate the protein phosphatase inhibitor DARRP-32 (Halpain et al., 1990), an important regulator of dopaminergic neurotransmission (Fienberg et al., 1998), whose activity may be necessary for the potentiation of NMDA receptors by protein kinase A (Blank et al., 1997). NMDA receptors participate in the regulation of striatal synaptic plasticity, through processes such as long-term potentiation of synaptic strength (Calabresi et al., 1992; Walsh and Dunia, 1993; Garcia-Munoz et al., 1996). Striatal lesioning with the NMDA agonist quinolinic acid mimics Huntington's disease (Beal et al., 1986, 1991; DiFiglia, 1990; Roberts et al., 1993; Figueredo-Cardenas et al., 1994), a hereditary neurodegenerative disorder that primarily affects striatal projection neurons and spares interneurons (Ferrante et al., 1987a,b; Reiner et al., 1988; Harrington and Kowall, 1991; Albin et al., 1992; Augood et al., 1996).

The NMDA receptor is thought to be a tetra- or pentamer (discussed in Dingledine et al., 1999) composed of subunit proteins from the NR1 (Moriyoshi et al., 1991) and NR2 families (Monyer et al., 1992). NR1 subunit proteins are abundantly expressed in all brain areas, and one NR1 subunit must be present for receptor function, at least in vitro. Expression of the four NMDAR2 receptor subunit proteins, NR2A–D, is regulated regionally and developmentally (Monyer et al., 1994; Sheng et al., 1994), and their presence or absence modulates physiologic and pharmacologic properties of the NMDA receptor complex (reviewed in Dingledine et al., 1999).

The mRNA encoding the NR1 subunit contains three alternatively spliced regions. The resulting NR1 subunit isoforms differ by the presence or absence of a segment in the amino-terminal region, termed N1, and by the expression or omission of the two independent protein segments, C1 and C2, at the carboxy-terminus of the protein. Omission of the region encoding C2 removes a stop codon and results in a new open reading frame, encoding the alternative carboxy-terminus C2′ (Fig. 1; Anantharam et al., 1992; Sugihara et al., 1992; Hollmann et al., 1993; Zukin and Bennett, 1995). NMDA receptors that contain different isoforms of NR1 differ in their affinity for glutamate, glycine, and NMDA (Durand et al., 1992). Alternative splicing of NR1 influences modulation of the receptor channel by polyamines (Durand et al., 1992; Traynelis et al., 1995), protons (Traynelis et al., 1995), divalent cations (Hollmann et al., 1993; Traynelis et al., 1998), Ca^{2+}/calmodulin (Ehlers et al., 1996; Hisatsune et al., 1997), and phosphorylation by protein kinases A and C (Durand et al., 1992; Tingley et al., 1993, 1997; Leonard and Hell, 1997; Grant et al., 1998; Logan et al., 1999). There is also evidence for a regulatory role of alternative splicing of NR1 in the subcellular localization of NMDA receptors (Ehlers et al., 1995), and splice segment-specific interactions have been reported with the cytoskeleton-associated protein yotiao (Lin et al., 1998) and the neurofilament subunit NF-L (Ehlers et al., 1998).

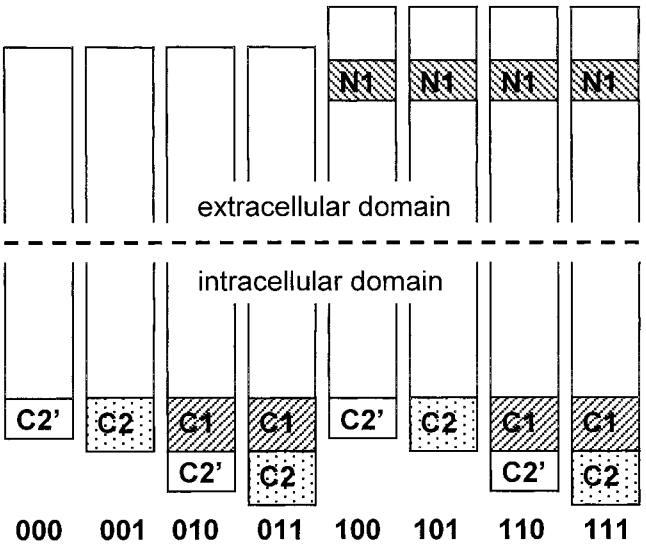

Fig. 1. Schematic of the eight possible NR1 receptor proteins generated by alternative splicing of *N*-methyl-D-aspartate receptor subunit R1 messenger RNA. Extracellular N- and intracellular C-terminals (Tingley et al., 1993) of the isoforms of NR1 are represented. The binary nomenclature (000–111) indicates the presence or absence of the three alternatively spliced segments N1, C1, and C2 (Zukin and Bennett, 1995). The membrane-spanning, conserved region of the protein is not illustrated. N1, which is a cassette of 21 amino acids located at the amino-terminus of the protein, is encoded by exon 5. Two independent consecutive splice cassettes encode the C1 and C2 segments of the protein, containing 37 and 38 amino acids, respectively. Excision of the segment encoding C2, which contains the stop codon, and splicing to an alternative downstream acceptor site result in the translation of 22 unrelated amino acids (C2′) before a second stop codon is reached (Hollmann et al., 1993).

Previous studies employing in situ hybridization have demonstrated the differential expression of the mRNAs encoding NR1 isoforms among different types of striatal neurons (Landwehrmeyer et al., 1995; Laurie et al., 1995). In an previous immunohistochemical study, we found that the striatal interneurons expressing nNOS contain an NR1 protein isoform distinct from those isoforms found in the majority of striatal cells (Weiss et al., 1998). In the present study, we have identified the NR1 protein isoforms present in projection neurons and several additional classes of interneurons in the striatum.

MATERIALS AND METHODS

Antibodies and antisera

All of the immunochemical reagents for the identification of alternatively spliced NMDAR1 segments employed in this study have been described previously in published studies (Table 1). Two affinity-purified antibodies, designated 22282 (for the N1 segment of NMDAR1) and 17182 (C1 segment of NMDAR1), were obtained from Dr. Morgan Sheng (Neurobiology Department and Howard Hughes

TABLE 1. Antibodies Used for Immunohistochemical Localization of NMDA Receptor Subunit Proteins*

Specificity	Identifier	Antigen	Amino acids	Dilution	Reference
NMDAR1-N1	22282	Peptide	1–21 of exon 5	0.5 μg/ml	Sheng et al. (1994)
NMDAR1-C1	17182	Peptide	864–900	0.5 μg/ml	Sheng et al. (1994)
NMDAR1-C2		Peptide	923–938	1:500	Aoki et al. (1994)
NMDAR1-C2′	D2 Ab	Peptide	1–17 of C2′	1:800	Iadarola et al. (1996)

*Listed are the specificity, identifying name or number, type of antigen, and working dilution for the *N*-methyl-D-aspartate (NMDA) receptor subunit protein antibodies used in this study. All antibodies were raised in rabbits. The references listed describe the production of these antibodies in more detail.

Medical Institute, Massachusetts General Hospital). These antibodies were produced by using peptide immunogens in rabbits and characterized by immunoblots and the ability to coimmunoprecipitate other NMDA subunits (Sheng et al., 1994). Use of the C1 segment antibody in previous immunohistochemical investigations has been reported (Liu et al., 1994; Johnson et al., 1996; Weiss et al., 1998). The antiserum to the C2 segment was obtained from Drs. Ted and Valina Dawson (Neurology and Neuroscience Department, Johns Hopkins University). It has been characterized by immunoblot analysis in brain extracts and transfected cells (Aoki et al., 1994) and used in several previous immunohistochemical studies (Aoki et al., 1994, 1997; Aoki, 1997; Weiss et al., 1998; Albers et al., 1999). When the C2 cassette of the NMDAR1 mRNA is absent, alteration of the reading frame produces a novel carboxy-terminal sequence (the "C2′ segment," Fig. 1; Huang et al., 1993; Iadarola et al., 1996). We used an antibody raised to a peptide and characterized both by Western blot and immunohistochemical techniques to localize this protein segment (Iadarola et al., 1996; Weiss et al., 1998; Albers et al., 1999). For neurochemical identification of striatal neurons, we used antibodies against calbindin (Sigma Chemicals, St. Louis, MO; diluted 1:100), parvalbumin (Sigma; diluted 1:1,000), calretinin (Chemicon International, Temecula, CA; diluted 1:2,000), ChAT (Chemicon; diluted 1:100), and nNOS. The nNOS antibody (JH8GP, diluted 1:1,000) was raised in guinea pig against a C-terminal peptide (amino acids 1413–1429) of rat nNOS and affinity purified. This antibody has been characterized in immunoblots of brain extracts and used in prior studies for immunohistochemical localization of nNOS (Huang et al., 1993 ; Aoki et al., 1997; Weiss et al., 1998).

Biotinylated dextran (BD) labeling

Retrograde tracing was conducted by using a modification of the methods of Rajakumar (1993) and Tallaksen-Greene (1994). Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital (100 mg/kg, i.p.) and placed in a stereotaxic frame. Biotinylated dextran (molecular weight = 10 kDA; Molecular Probes Inc., Eugene, OR), freshly dissolved in double distilled water (5%), was pressure injected (2 μl; 1 μl/minute) with a syringe (30 gauge; Hamilton Co., Reno, NV) into the substantia nigra (n = 4) and globus pallidus (n = 4) by using coordinates taken from the atlas of Paxinos and Watson (1986). For the substantia nigra, coordinates were: AP −4.4, ML +2.0, DV −8.0 from bregma. For globus pallidus, the needle was tilted 16° medially to avoid penetrating the striatum and coordinates were: AP −0.8, ML +0.9, DV −6.5 from bregma. Animals were allowed to survive for 10–12 days.

Tissue processing

In all studies, the animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused with normal saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS), at room temperature (RT). The brains were removed immediately, postfixed for 1 hour in the same fixative at RT, and then cryoprotected overnight in 30% sucrose at 4°C. The brains were then frozen in isopentane cooled with dry ice, and 50-μm coronal sections were cut with a freezing microtome. The sections were then either processed immediately for immunohistochemistry or stored in 50% glycerol in 100 mM Tris, pH 7.5, at −20°C. All animal-related procedures were performed in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Immunohistochemistry

Dual-label immunohistochemistry was conducted as described previously (Standaert et al., 1986; Testa et al., 1998). BD-injected brains were processed for single-label immunohistochemistry. In all studies, the sections were washed in PBS and then incubated in 3% normal goat serum or 3% normal donkey serum with 0.3% Triton X-100 in PBS for 1 hour. For dual-label immunohistochemistry, sections were incubated over two nights at RT (for C2′ segment antibodies) or 4°C (all others) in the same solution containing combinations of primary antibodies diluted as listed in Table 1 with antibodies against calbindin, parvalbumin, calretinin, ChAT, or nNOS. Sections were then washed in PBS and incubated sequentially in fluorescent secondary antibodies. Antibodies to NMDA subunits were visualized by using goat anti-rabbit or donkey anti-rabbit antiserum coupled to Cy3 (Jackson Laboratories, West Grove, PA; diluted 1:200). Neuronal calbindin, parvalbumin, and ChAT staining was visualized with goat anti-mouse serum coupled to Cy2 (Jackson Laboratories; diluted 1:800). Calretinin staining was visualized with donkey anti-goat antiserum labeled with fluorescein isothiocyanate (FITC; Jackson Laboratories; diluted 1:50). Neuronal NOS staining was visualized with goat anti-guinea pig antiserum labeled with FITC (Jackson Laboratories; diluted 1:50). Sections for combined retrograde tracing and immunohistochemistry were incubated over two nights with primary antibodies, as listed in Table 1. NMDA subunit receptor antibodies were detected by using goat anti-rabbit antiserum coupled to Cy3 (1:200; Jackson Laboratories), followed by incubation in FITC-labeled avidin (1:500; Jackson Laboratories) for 1 hour to visualize biotin-labeled neurons. All sections were then washed in PBS, mounted on gelatin-coated slides, dried, and coverslipped using glycerol containing 100 mM Tris, pH 8.0, and 0.2% *p*-phenylenediamine (Sigma) to retard fading. Each experiment included control tissue, processed with omission of one or both primary antibodies.

Preparations were examined using a Bio-Rad Laser Confocal system (MRC 1024, Richmond, CA) equipped with a Leica DMRB microscope and an argon/krypton laser. Images were obtained by illuminating the section with a single laser line and collecting the image by using an appropriate emission filter: for Cy3, excitation at 568 nm and a 605-nm longpass filter; for FITC, excitation at

488 nm and a 522 nm bandpass filter. For each wavelength, four sequential images 1,024 × 1,024 pixels in size with an 8-bit pixel depth were obtained and averaged by using a Kalman filtering method to reduce noise. Images were assembled for display and contrast adjusted in Adobe Photoshop. No further image processing was performed.

Double-labeled neurons were counted manually with a Leica DMRB microscope equipped with N2 and I2 filter sets, in two coronal sections of two different rat brains per double-label combination. Cells were counted in the left and right striata in three dorsoventral columns located in the medial, central, and lateral striata. The intensity of NR segment immunoreactivity within each cell was graded in four categories, as described by Kosinski et al. (1997): 0, immunonegative: area occupied by the cell was unstained, e.g. Figure 2B2 and 2B5 (arrows); +, low: a small number of puncta was visible throughout the cytoplasm of the cell, e.g., Figure 3G (arrow); ++, moderate: small clusters of staining were arrayed along the margins of the cell, e.g., Figure 2A2 (cells not indicated by arrows or arrowheads); and +++, intense: clusters of staining formed a continuous ring around the cell, e.g., Figure 2A2 and 2A5 (arrows).

RESULTS

Striatum displays distinct patterns of NMDAR1 receptor isoform immunoreactivity

The antibodies targeted to segments encoded by alternatively spliced regions of the NMDAR1 mRNA each produced distinct patterns of immunoreactivity in rat striatum. Only a few NMDAR1-N1-immunopositive neurons were detected, and these displayed staining of the cytoplasm and membrane of the soma and proximal dendrites (Fig. 3). The antiserum against the N1 segment also labeled numerous small puncta in the neuropil, and occasionally there were linear aggregations of immunoreactivity resembling beads on a string. NMDAR1-C1-immunopositive neurons were abundant and displayed intense membrane and moderate cytoplasmic staining (Figs. 2A, 4). Immunoreactivity for C1 was stronger in the medial and ventral regions of the striatum. The antiserum against the C1 segment produced moderate staining in the neuropil. The vast majority of neurons present in the striatum displayed moderate C2 segment immunoreactivity. This antiserum also produced low intensity staining of small puncta in the striatal neuropil (Fig. 6). The NMDAR1-C2′ antiserum labeled a small number of cells, most of which exhibited strong staining (Fig. 7), whereas the neuropil remained largely unstained. In the absence of primary antisera, no staining was detectable within cells or in the neuropil, and the staining patterns were not altered by coincubation with the neuronal marker antibodies.

Identification of neostriatal neurons

The antiserum against calbindin D_{28K} stained a large number of striatal neurons and striatal neuropil in a patchy distribution (Fig. 2A). Previous studies have demonstrated that the calbindin-poor regions correspond to the striatal striosomal compartment, whereas the intensely stained regions correspond to the matrix compartment (Gerfen et al., 1985; Graybiel, 1990; Gerfen, 1992). The antisera against striatal interneurons, targeted to parvalbumin, calretinin, ChAT, and nNOS, each stained a modest number of striatal cells consistent with the low abundance of interneurons in the striatum (Kawaguchi et al., 1995). Parvalbumin cells were often slightly larger than those labeled by calbindin and were polygonal, oval, or fusiform in shape, as previously described (Cowan et al., 1990; Kita et al., 1990; Fig. 2B). ChAT-immunopositive cell soma were large, and the staining was found throughout the cytoplasm, as previously described (Bolam et al., 1984; Figs. 3C, 4C, 6C, 7C). The antibody to calretinin stained a modest number of small striatal neurons, consistent with previous descriptions (Bennett and Bolam, 1992; Figs. 3D, 4D, 6D, 7D). The antibody to nNOS labeled cell bodies and extensive neuronal arborization, as reported in a previous study (Weiss et al., 1998; data not shown). Injections of BD into the substantia nigra pars reticulata (SNr) and in the globus pallidus (GP) resulted in retrograde labeling of numerous medium-sized striatal neurons and their dendritic processes in the ipsilateral striatum (Fig. 5A,B).

Localization of NMDAR-N1 immunoreactivity in identified striatal neurons

Calbindin-immunolabeled striatal projection neurons (Fig. 3) and those identified by retrograde tracing from the SNr and GP (data not shown) displayed only a very low level of N1 segment immunoreactivity, similar to that present in the neuropil. Calretinin-, ChAT- and nNOS-immunopositive neurons likewise contained very low levels of N1 segment immunoreactivity. Two-thirds (31/45, Table 2) of the parvalbumin neurons, however, displayed moderately strong staining for the N1 segment of NMDAR1 (Fig. 3).

Localization of NMDAR-C1 immunoreactivity in identified striatal neurons

Calbindin neurons exhibited strong staining with the antibody to the NMDAR1-C1 segment. Of 55 consecutive calbindin-positive cells examined, 30 displayed moderate and 22 intense immunoreactivity for the C1 segment (Figs. 2A, 4), whereas only three had no detectable C1 staining (Table 2). In addition, there was a modest number of medium-sized neurons labeled by the C1 antibody that did not stain for calbindin (Fig. 2A); most of these neurons were found in the calbindin-poor striosomal regions. All of the striatofugal neurons detected by injection of retrograde label in the SNr and most of those detected by injection in the GP were C1 segment immunopositive (Fig. 5). None of the other populations of striatal neurons examined displayed C1 segment immunoreactivity (Fig. 4, Table 2).

Localization of NMDAR-C2 immunoreactivity in identified striatal neurons

All neuronal populations of the striatum, including projection and interneurons, displayed C2 segment immunoreactivity (Fig. 6). Although most striatal neurons contained moderate amounts of immunoreactivity, the majority of nNOS-immunopositive neurons (35 of 53) exhibited low immunolabeling (Table 2).

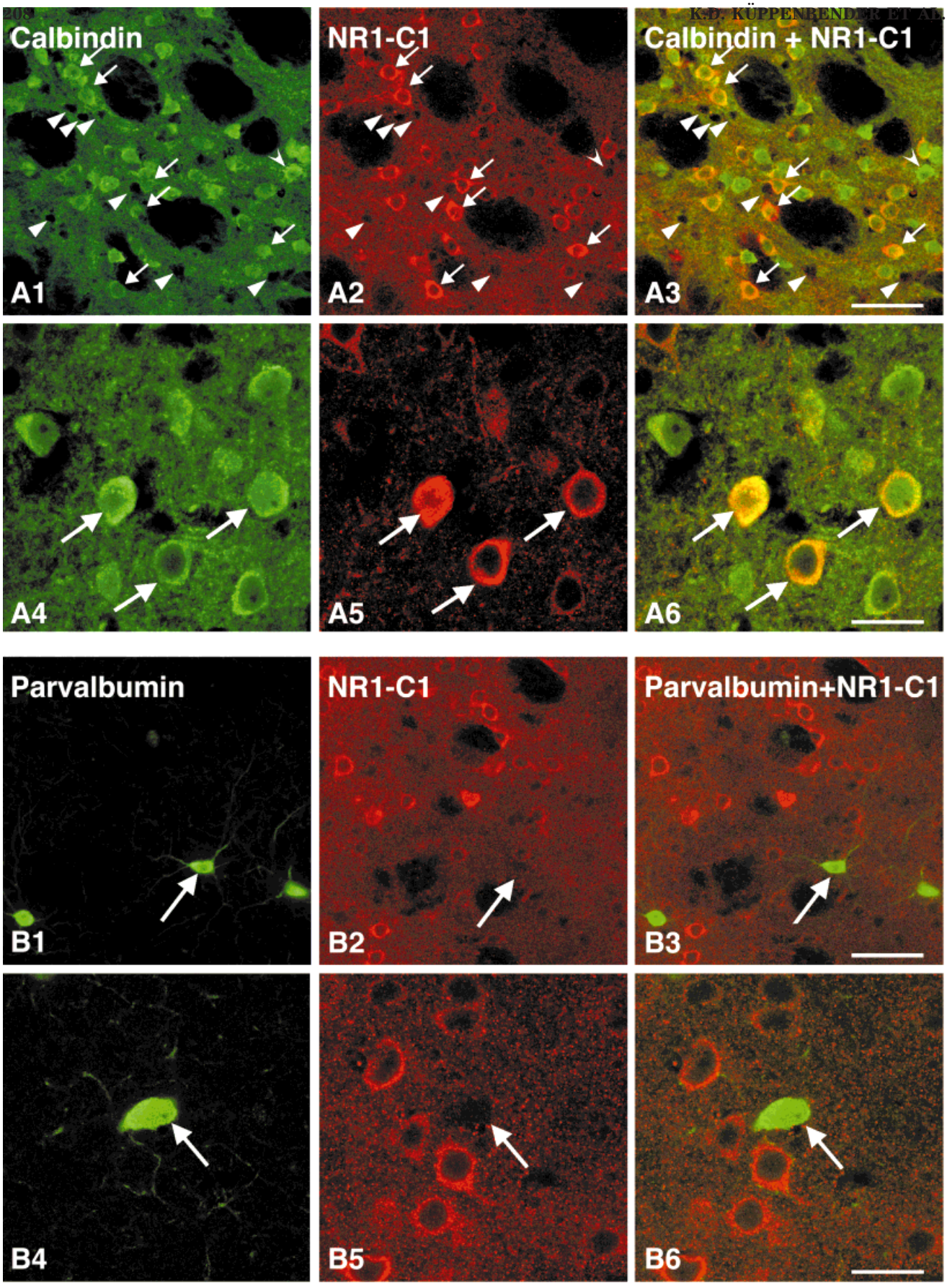
Calbindin
NR1-C1
Calbindin + NR1-C1
A1
A2
A3
A4
A5
A6
Parvalbumin
NR1-C1
Parvalbumin+NR1-C1
B1
B2
B3
B4
B5
B6

Figure 2

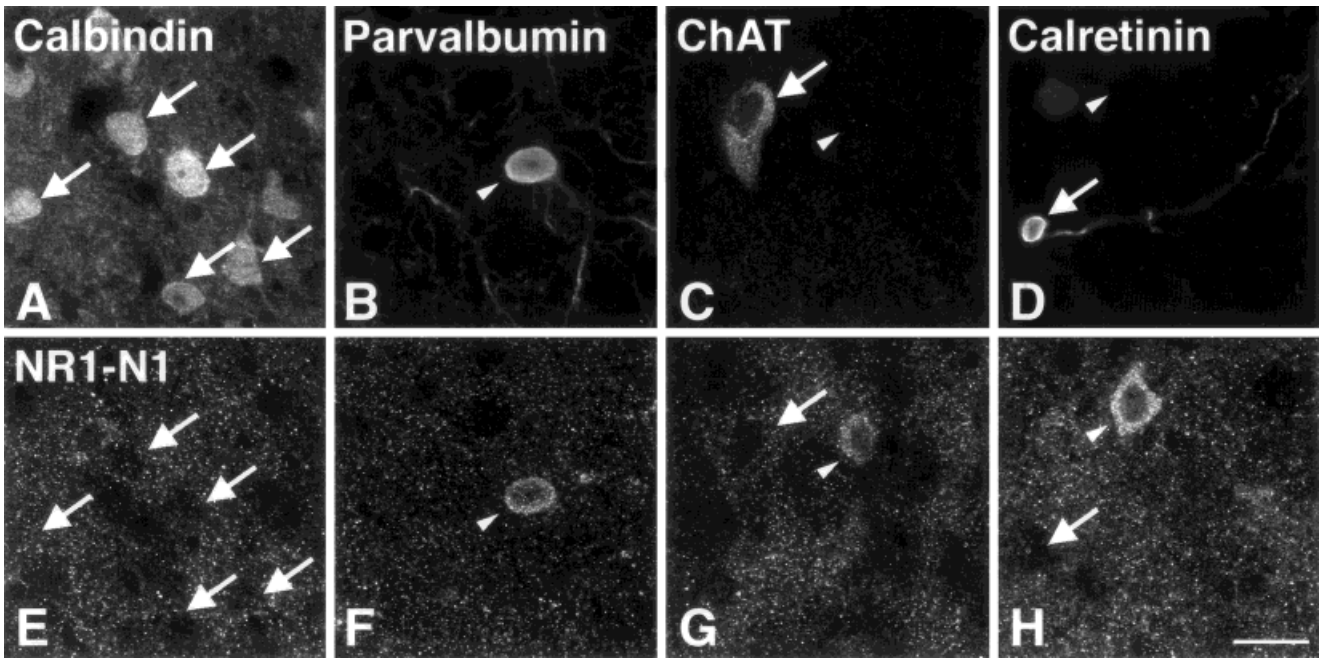

Fig. 3. Localization of *N*-methyl-D-aspartate receptor subunit R1 isoforms that contain the N1 segment in identified striatal neurons. **A–D:** Images show cells immunopositive for calbindin, parvalbumin, choline acetyltransferase (ChAT), and calretinin. C: ChAT cells are larger than all other cells. D: The calretinin cell displays a long, immunoreactive process. **E–H:** Images show N1 segment immunoreactivity of the same areas. The pattern of staining is punctuate and relatively low in intensity. Arrowheads indicate N1-immunopositive cells, which colocalize with parvalbumin (B,F) but not with calbindin, ChAT, or calretinin cells (arrows). Scale bar = 20 μm.

Localization of NMDAR-C2′ immunoreactivity in identified striatal neurons

Immunoreactivity for the C2′ segment was virtually absent from calbindin-immunoreactive neurons (Fig. 7, Table 2) and retrogradely labeled striatofugal projection neurons (data not shown). Conversely, the majority of the striatal interneurons contained C2′ segment immunoreactivity. The most intense staining was found over nNOS-positive neurons, as described previously (Weiss et al., 1998). Parvalbumin- and calretinin-immunoreactive neurons both displayed moderate C2′ segment labeling, whereas ChAT-immunopositive neurons showed low or no C2′ staining (Fig. 7).

Fig. 2. Localization of NR1-C1 splice segment immunoreactivity in striatal neurons stained for calbindin and parvalbumin. **A1** and **A4** illustrate staining for calbindin in striatal neurons and neuropil. Unstained areas represent tracts of white matter fibers. **A2** and **A5** illustrate C1 immunoreactivity in the same cells, whereas **A3** and **A6** are superimpositions of these images. Many but not all calbindin neurons were double labeled. The intensities of C1 immunoreactivity differed. The arrows point to intensely (+++) C1-immunoreactive cells. The closed arrowheads indicate calbindin-negative cells, which were also C1 immunonegative. The open arrowhead indicates a calbindin-negative cell that displayed C1 segment staining. **B1** and **B4** illustrate the less prevalent parvalbumin-immunopositive cells, demonstrating labeling of cell body and processes. **B2** and **B5** illustrate C1 segment immunoreactivity, and **B3** and **B6** are superimpositions of these images. Arrows point to parvalbumin cells and indicate the absence of C1 immunoreactivity over these cells. Scale bars = 50 μm in A3,B3 also apply to A1,A2,B1,B2; 20 μm in A6,B6 also apply to A4,A5,B4,B5.

DISCUSSION

By using antibodies selective for the three alternatively spliced segments of the NMDAR1 receptor protein, we examined the localization of NMDAR1 receptor isoforms in rat striatal neurons. Projection neurons were identified by staining for calbindin D_{28K} and by retrograde tracing from the targets, the GP and SNr. We found that these projection neurons are characterized by intense staining for the C1 segment, a property that distinguishes them from each of the several types of interneurons examined. Interneurons containing the calcium-binding proteins parvalbumin or calretinin and those expressing nNOS exhibit a different pattern of staining for the NMDAR1 isoforms: they do not stain for the C1 region but do stain intensely for the alternative C2′ terminus of NMDAR1. Cholinergic interneurons, identified by the presence of ChAT immunostaining, also lack staining for the C1 segment but differ from the other interneuron types in that they are only weakly immunoreactive for the C2′ segment.

Technical considerations

The antibodies to NMDAR1 used in our study were directed to peptide or fusion protein epitopes from discrete regions of the NMDAR1 subunit protein. They were targeted to the alternatively spliced segments of the NMDAR1 subunit and produced regional patterns of staining that are consistent with the known distribution of mRNAs encoding these segments (Laurie and Seeburg, 1994b; Standaert et al., 1994; Landwehrmeyer et al., 1995). Each of the antibodies stained neuronal membranes, cytoplasm, and the neuropil in a fine, granular pattern, whereas the nuclei remained unstained. These findings are consistent

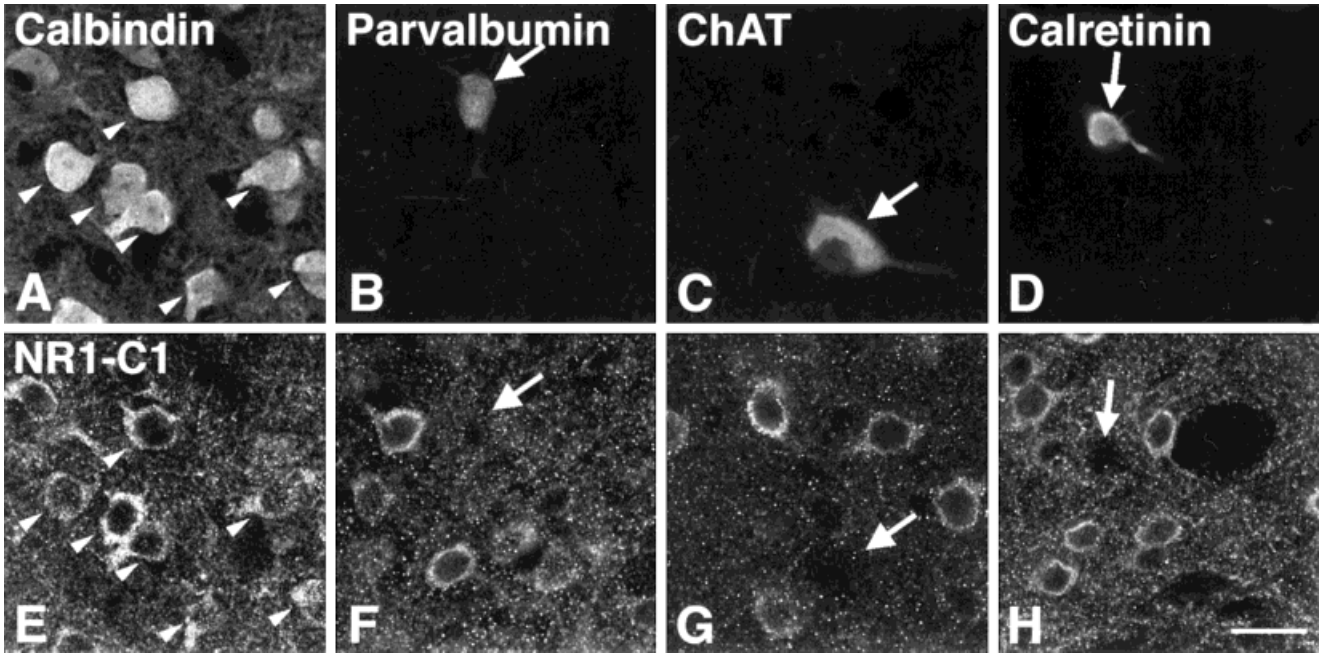

Fig. 4. Localization of the C1 segment of *N*-methyl-D-aspartate receptor subunit R1 in identified striatal neurons. **A–D:** Images show cells immunopositive for calbindin, parvalbumin, choline acetyltransferase (ChAT), and calretinin. **E–H:** Images show C1 segment immunoreactivity in the same areas. A,E: Arrowheads indicate calbindin-immunopositive neurons, most of them displaying moderate or intense C1 staining. Arrows indicate all other cells that appear as areas of diminished immunoreactivity compared with the surrounding neuropil. Scale bar = 20 μm.

with ultrastructural studies that have demonstrated clusters of NMDAR1 immunoreactivity both at sites of synaptic contact and within the cytoplasm of somata and dendrites (Aoki et al., 1994; Huntley et al., 1994; Petralia et al., 1994; Siegel et al., 1994; Farb et al., 1995; Gracy and Pickel, 1995; Johnson et al., 1996; Bernard and Bolam, 1998). The antibody to the C2 region displayed prominent immunoreactivity in the neuropil, as previously described by others using the same (Weiss et al., 1998) or a different (Petralia et al., 1994; Farb et al., 1995) antibody to the C2 region. It is important to note that the antisera distinguish epitopes associated with different regions of the NMDAR1 protein, but it is not possible to determine with these anatomical methods whether these epitopes are associated with intact, full-length NMDAR1 transcripts.

The immunochemical markers that we used to detect striatal interneurons reproduced the known morphology and patterns of distribution (Armstrong et al., 1983; Vincent and Johansson, 1983; Bolam et al., 1984; Cowan et al., 1990; Kita et al., 1990; Dawson et al., 1991; Jacobowitz and Winsky, 1991; Bennett and Bolam, 1992; Kawaguchi et al., 1995; Weiss et al., 1998). Previous studies have examined the overlap between these markers and other neurotransmitters known to be expressed by striatal neurons. The neurons that contain parvalbumin, calretinin, and nNOS are GABAergic, and most of them contain the 67-kD form of glutamic acid decarboxylase (Cowan et al., 1990; Kubota et al., 1993; Lenz et al., 1994). Neuronal NOS interneurons, which can be identified histologically by the presence of nicotinamide adenine dinucleotide phosphate (NADPH-) diaphorase activity (Dawson et al., 1991), also contain somatostatin and neuropeptide Y (Vincent and Johansson, 1983; Rushlow et al., 1995). Some but not all nNOS interneurons also stain for calbindin D_{28K} (Bennett and Bolam, 1993).

Colocalization of the NMDAR1 epitopes with the neuronal markers was examined by using confocal microscopy with parameters that allowed for very thin optical sections (approximately 1 μm), thereby largely avoiding the confounding influence of overlying structures. With this method, we were able to clearly visualize staining for the NMDAR1 epitopes within the perikarya of labeled neurons. Nevertheless, the spatial resolution of this optical method was not sufficient to determine the localization of the immunoreactivity found in the neuropil or accurately determine the relation of this neuropil staining to the processes of striatal neurons. Thus, our analysis is necessarily limited to the NMDAR1 proteins associated with the soma, and we cannot exclude the possibility that some epitopes may be largely absent from this compartment yet still present within the peripheral processes of the cells.

NR1-C1 segment is confined to striatal projection neurons

Calbindin D_{28K}, a calcium-binding protein, is enriched in the matrix compartment of the striatum. It is contained in medium-sized spiny projection neurons (Gerfen et al., 1985; DiFiglia et al., 1989) and in a small population of aspiny neurons recognized as a subset of NADPH-diaphorase/nNOS interneurons (Kiyama et al., 1990; Bennett and Bolam, 1993). We found that the vast majority of calbindin-D_{28K}-positive neurons displayed strong C1 segment immunoreactivity. Because calbindin staining is not found in projection neurons within the striatal striosomes and does not distinguish between striatopallidal and striatonigral cells, we also employed retrograde tracing to

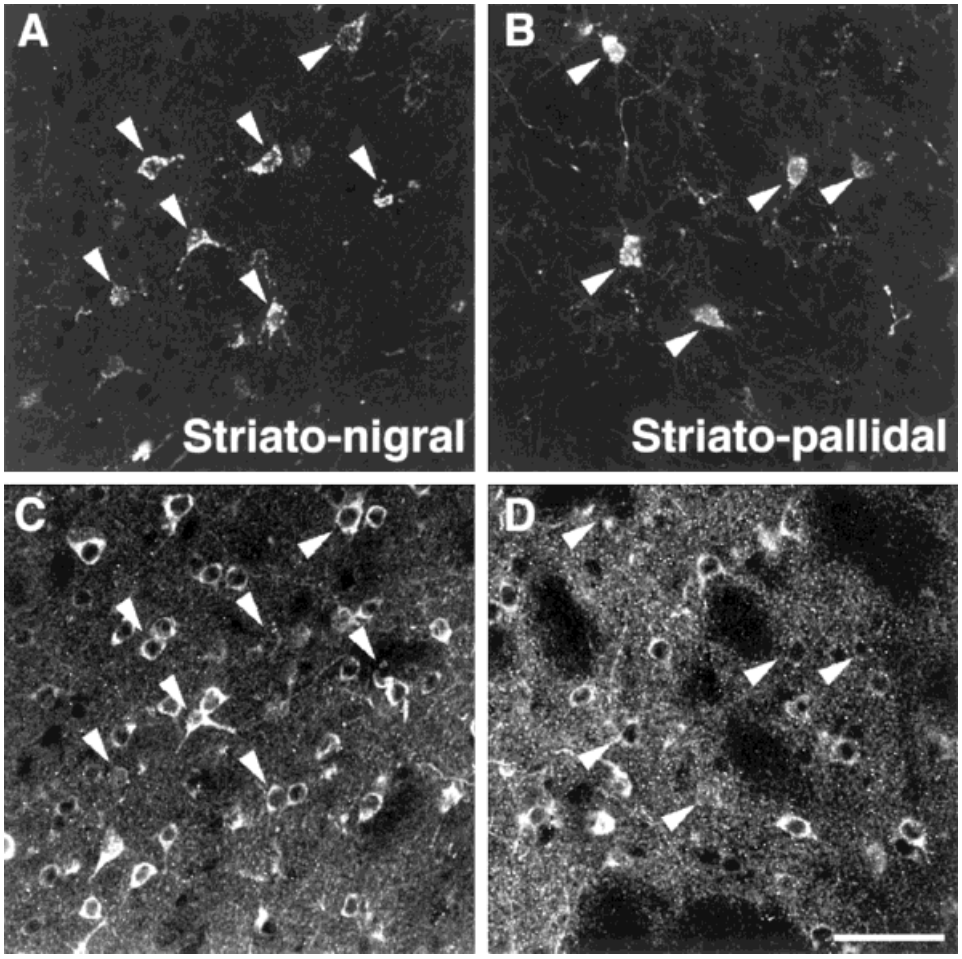

Fig. 5. Localization of C1 segment immunoreactivity in striatonigral and striatopallidal projection neurons. The upper row shows striatonigral (**A**) and striatopallidal (**B**) projection neurons identified by retrograde tracing. The lower row shows C1 segment immunoreactivity in the same areas (**C,D**). Arrowheads indicate projection neurons. Neurons in both striatofugal populations display C1 segment immunoreactivity. Scale bar = 50 μm.

TABLE 2. Intensity of NR1 Splice Segment Immunolabeling of Identified Striatal Neurons*

NR1 segment immunostaining	Cell count				
	Calbindin	Parvalbumin	Calretinin	ChAT	nNOS
N1					
0	0	0	0	0	0
+	50	14	46	63	49
++	0	31	0	0	2
+++	0	0	0	0	0
C1					
0	3	42	51	58	51
+	0	0	8	0	0
++	30	0	0	0	0
+++	22	0	0	0	0
C2					
0	0	0	0	0	0
+	0	0	0	0	35
++	50	50	50	50	18
+++	0	0	0	0	0
C2′					
0	55	0	0	23	0
+	0	0	8	31	0
++	0	33	89	0	5
+++	1	43	9	0	91

*The intensities of NR1 segment immunolabeling in striatal neurons identified by the neurochemical markers calbindin, parvalbumin, choline acetyltransferase (ChAT), and neuronal nitric oxide synthase (nNOS) were graded in four categories: 0, immunonegative; +, low; ++, moderate; +++, intense. The figures indicate neurons counted in coronal sections from two different rat brains.

identify projection neurons. With this approach, we found that neurons retrogradely labeled from either the GP or the SNr both displayed intense C1 immunoreactivity. These findings suggest that striatal neurons projecting to the SNr and those projecting to the GP both contain the C1 segment. It should be noted, however, that most of the axons of striatal neurons projecting to the SNr pass through the GP, where in rat up to 40% of striatonigral axons give off minor collaterals to the GP (Loopuijt and van der Kooy, 1985). Because we used pressure injection of the tracer, some striatonigral neurons may have been labeled by uptake into fibers of passage by pallidal injections and some striatopallidal neurons may have been labeled as a result of uptake from their nigral collaterals. These potential issues do not alter the conclusion that C1 staining is associated exclusively with projection neurons, but they do suggest caution in comparing the relative extent of labeling seen with the pallidal and nigral injections. The selective localization of the C1 segment in projection neurons is consistent with results from a previous study employing in situ hybridization, in which the mRNA encoding the C1 segment was detected in striatal enkephalinergic projection neurons but not in interneurons (Landwehrmeyer et al., 1995).

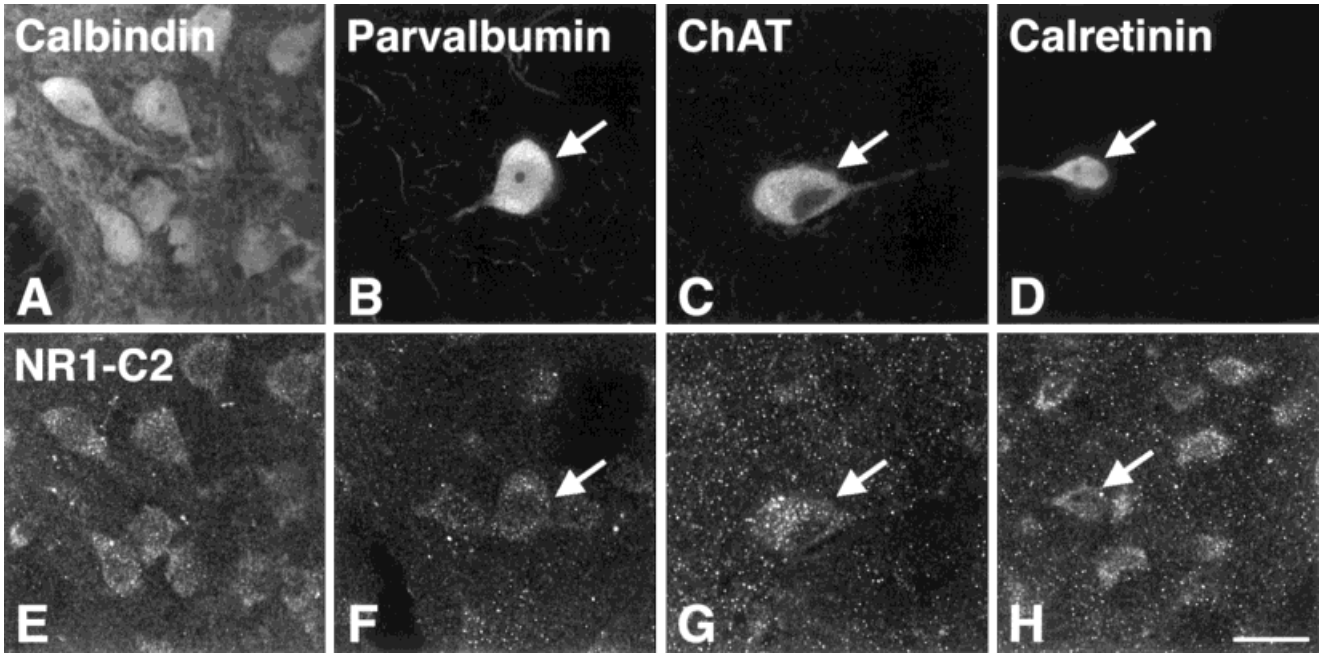

Fig. 6. Localization of the C2 segment of *N*-methyl-D-aspartate receptor subunit R1 in identified striatal neurons. **A–D:** Images show cells immunopositive for calbindin, parvalbumin, choline acetyltransferase (ChAT), and calretinin. **E–H:** Images show C2 segment immunoreactivity in the same cells. All cells display moderate C2 staining. Arrows indicate parvalbumin, ChAT, and calretinin cells. Scale bar = 20 μm.

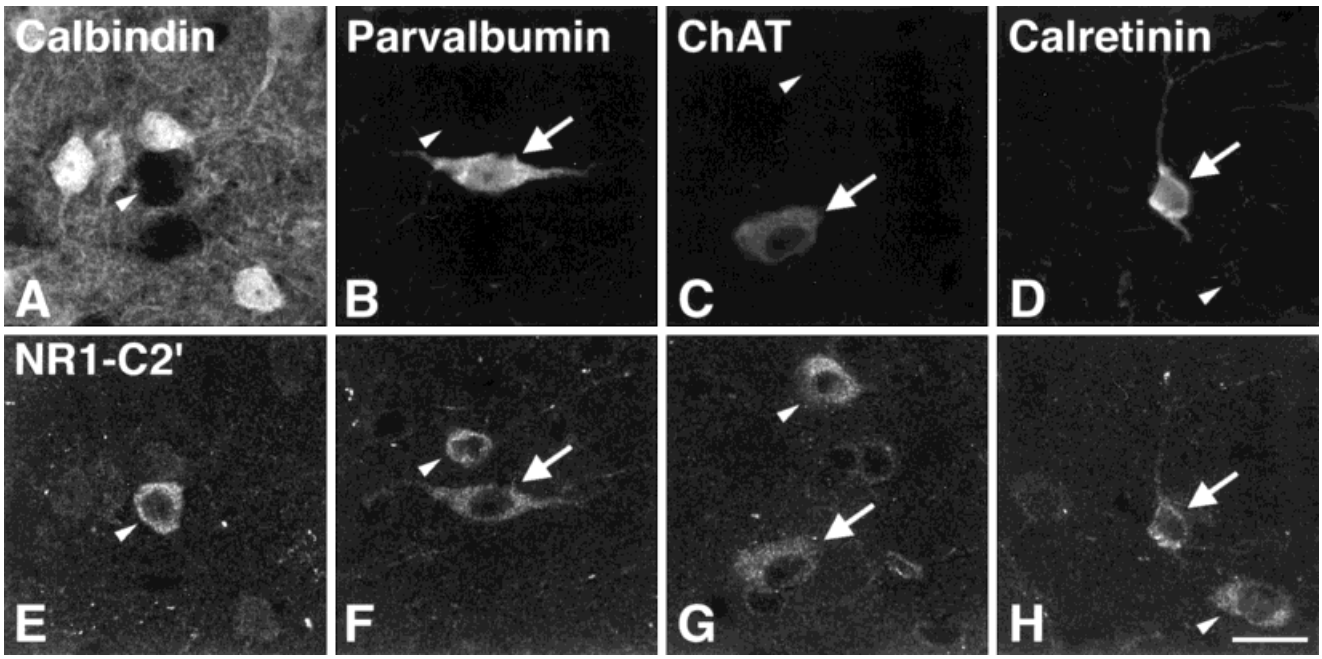

Fig. 7. Localization of the C2′ segment of *N*-methyl-D-aspartate receptor subunit R1 in identified striatal neurons. **A–D:** Images show cells immunopositive for calbindin, parvalbumin, choline acetyltransferase (ChAT), and calretinin. **E–H:** Images show C2′ segment immunoreactivity in the same areas. Calbindin cells display no C2′ immunoreactivity, whereas calretinin, parvalbumin, and ChAT cells exhibit C2′ staining (arrows). The arrowheads indicate C2′-immunopositive cells that are not labeled by calbindin, parvalbumin, ChAT, or calretinin, respectively. There is very little labeling of the surrounding neuropil. Scale bar = 20 μm.

The C1 segment contains residues that are targets for phosphorylation, and it is important for the regulation of subcellular localization of NMDA receptor complexes. Binding of Ca^{2+}/calmodulin to a site within the C1 segment reduces the channel open rate (Ehlers et al., 1996), and this binding is inhibited by protein kinase C–dependent

phosphorylation of the C1 segment (Tingley et al., 1993; Hisatsune et al., 1997; Leonard and Hell, 1997; Chakravarthy et al., 1999). The C1 segment also contains a phosphorylation site specific for protein kinase A (Tingley et al., 1997), and activation of protein kinase A enhances the current response of the NMDA receptor in *Xenopus* oocytes injected with rat striatal poly(A^+) mRNA (Blank et al., 1997). NR1 isoforms that contain the C1 segment form membrane-associated clusters in transfected fibroblasts, whereas other NR1 isoforms are distributed throughout cytoplasm of the cells. Phosphorylation of a serine residue within the C1 segment by protein kinase C rapidly and reversibly disperses the clusters into the cytoplasm (Ehlers et al., 1995; Tingley et al., 1997). These effects may result from interaction of the C1 segment with the cytoskeletal proteins, including yotiao (Lin et al., 1998) and the neurofilament subunit NF-L (Ehlers et al., 1998).

The alternative NR1 carboxy-terminal C2′ is confined to striatal interneurons

The alternative splicing of the 3′ region of the NMDAR1 mRNA results in a receptor subunit terminating in one of two alternative endings, termed C2 and C2′. By using an antibody to the C2 segment, we have found that this epitope is present in all the types of striatal cells examined and is also fairly abundant in the striatal neuropil. In contrast, the C2′ segment is selectively accumulated by some but not all striatal interneurons. In particular, nNOS neurons are stained intensely for the C2′ segment, and these cells show lower immunoreactivity for the C2 segment. Interneurons containing the calcium-binding proteins parvalbumin and calretinin also exhibit strong staining for the C2′ segment. Interestingly, cholinergic interneurons contain only low levels of immunoreactivity for the C2′ epitope.

The functional role of the C2 and C2′ segments are incompletely defined, but one potentially important action is to regulate the cellular localization of the receptor assemblies. The C2′ segment contains a sequence for anchoring to the postsynaptic density protein 95 (Kornau et al., 1995). Whereas NR1 isoforms containing the C2 terminus are found in an unassembled, monomeric form in mouse brain, those with the alternative C2′ terminus are clustered with other subunits (Chazot and Stephenson, 1997). In a recent study employing immunoprecipitation, the C2 and C2′ segments were precipitated together from homogenized rat forebrain, suggesting that single receptor assemblies may contain NR1 subunits with each of the two termini (Blahos and Wenthold, 1996).

The NR1-N1 segment is confined to parvalbumin interneurons

Cellular labeling for the N1 segment in the striatum was very rare and confined to a heteromorphic subgroup of neurons comprising about two-thirds of the parvalbumin interneurons. The striatal neuropil displayed moderately dense N1 segment immunoreactivity. In a previous study using a different antibody to the N1 region together with tissue pretreatment with hydrogen peroxide and sodium hydroxide, Nash et al. (1997) detected striatal N1 segment labeling in unidentified medium-sized round and some scattered large neurons. These could in part correspond to the N1 segment immunopositive parvalbumin interneurons we observed. However, our findings indicate that the largest population of medium-sized striatal neurons, i.e., the medium spiny projection neurons, do not contain NR1 isoforms with the N1 segment. This conclusion is consistent with the known distribution of exon 5 mRNA. When using in situ hybridization, only very low levels of the mRNA for this segment are found in the rat striatum (Standaert et al., 1994; Laurie et al., 1995), and a dual label in situ hybridization study by Landwehrmeyer et al. (1995) detected no significant expression of the exon 5 insert in striatal enkephalin containing projection neurons, cholinergic, or somatostatin/nNOS interneurons.

The N1 segment appears to have an important effect on the agonist binding properties of NMDA channels. Presence of the N1 segment reduces the current response of the NMDA receptor to glutamate, glycine, and NMDA (Durand et al., 1992; Hollmann et al., 1993) and inhibits the potentiation by polyamines (Durand et al., 1992; Traynelis et al., 1995) and by micromolar concentration of Zn^{2+} (Hollmann et al., 1993; Traynelis et al., 1998). Presence of the N1 segment also potentiates the receptor through relief from proton inhibition (Traynelis et al., 1995).

NMDAR1 isoforms in striatal neurons

Our data lead us to the conclusion that projection neurons and interneurons contain different NR1 isoforms. The antibodies we employed recognize epitopes within the alternatively spliced regions. Thus, determining the isoforms present requires assuming that the segments are in fact present within intact NMDAR1 subunits. Projection neurons appear to lack the N1 segment and the alternative C2′ carboxy-terminus. Although virtually all projection neurons displayed C1 segment immunoreactivity, these neurons may also contain NR1 isoforms without the C1 segments. Thus the predominant NR1 isoforms in projection neurons would be $NR1_{011}$ and possibly $NR1_{001}$ (Fig. 8), using the terminology of Zukin and Bennett (1995; Fig. 1).

Parvalbumin-, calretinin-, and nNOS-containing interneurons appear to lack the C1 segment but contain both types of carboxy-termini, i.e., the C2 and the C2′ segments. A subgroup of parvalbumin interneurons appears to contain NR1 isoforms with an N1 segment. Therefore, the predominant NR1 isoforms of calretinin/GABAergic and somatostatin/nNOS interneurons would be $NR1_{000}$ and $NR1_{001}$ (Fig. 8). In addition, parvalbumin interneurons may contain the isoforms $NR1_{100}$ and $NR1_{101}$. Cholinergic interneurons appear to contain C2 segments but very little C2′ segment immunoreactivity. They also lack N1 and C1 segments and therefore most likely contain predominantly the $NR1_{001}$ isoform (Fig. 8).

Consequences for selective vulnerability

Calbindin-D_{28K}-immunoreactive neurons selectively degenerate in Huntington's disease (Seto-Ohshima et al., 1988; Kiyama et al., 1990), whereas interneurons are spared. Striatal injection of the NMDA-agonist quinolinic acid imitates this pattern of neuronal cell death in rats, suggesting a role for the NMDA receptor in the pathogenesis of Huntington's disease (Beal et al., 1986, 1991; DiFiglia, 1990; Roberts et al., 1993; Figueredo-Cardenas et al., 1994). Because all known populations of striatal neurons contain NMDA receptors (Chen et al., 1996; Küppenbender et al., 1997; Standaert et al., 1999), differences in the subunit composition among distinct neuronal populations may play a role in the selective vulnerability of striatal cells to injury or disease processes. The exclusive localization of the C1 segment to calbindin-D_{28K}-immuno-

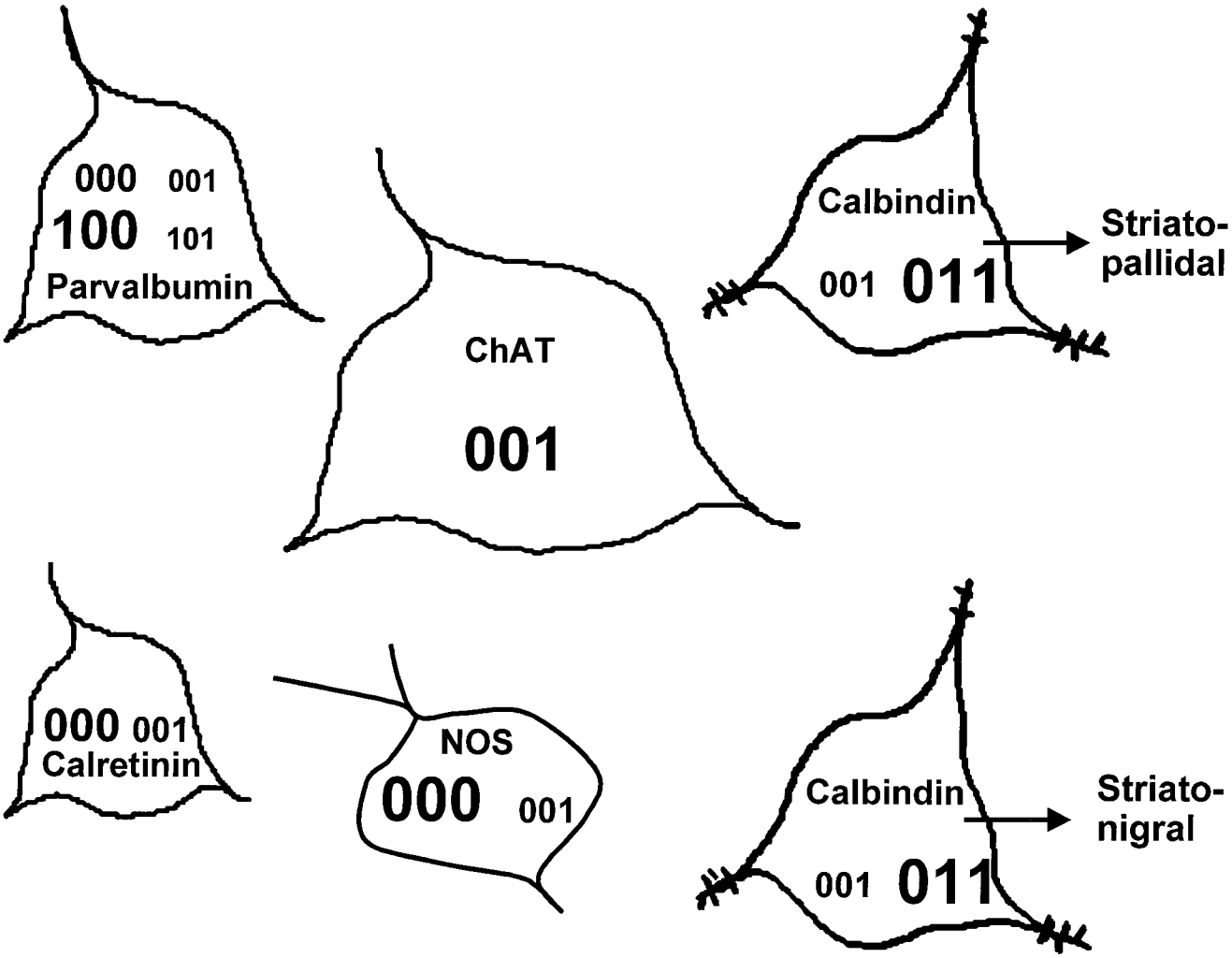

Fig. 8. Schematic of the putative distribution of alternatively spliced NR1 isoforms in rat neostriatal neurons. The isoforms are represented with a binary nomenclature (000–111) indicating the presence or absence of the various segments, as shown in Figure 1. The predominant isoform contained in each neuronal population is indicated by a larger font.

reactive projection neurons may have an important influence on the vulnerability of these cells, perhaps because of presence of the regulatory phosphorylation sites in this region or the role of the C1 segment in targeting NMDAR1 to synaptic sites. The C1 segment may also participate in dopamine-mediated regulation of NMDA receptor function (Cepeda et al., 1993; Snyder et al., 1998), and dopamine D1 receptor activation enhances the neurotoxic effect of NMDA on medium-sized striatal neurons (Cepeda et al., 1998). In vivo, NR1 isoforms combine with NR2A–D subunits to form receptors with distinct functional properties (Buller et al., 1994; Laurie and Seeburg, 1994a; Gallagher et al., 1997; Williams, 1997; Calabresi et al., 1998; Krupp et al., 1998; Traynelis et al., 1998). Projection neurons contain only NR2A and NR2B subunits, whereas interneurons also contain NR2D subunits (Landwehrmeyer et al., 1995; Standaert et al., 1996, 1999). Further studies of the functional properties of these specific receptor subunit combinations may help to elucidate the basis for selective vulnerability of the different types of striatal neurons.

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